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Identification and expression analysis of two small heat shock protein cDNAs from developing seeds of biodiesel feedstock plant *Jatropha curcas*

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ABSTRACT

Plant small heat shock proteins (sHSPs) are known to be important for environmental stress tolerance and involved in various developmental processes. In this study, two full-length cDNAs encoding sHSPs, designated *JcHSP-1* and *JcHSP-2*, were identified and characterized from developing seeds of a promising biodiesel feedstock plant *Jatropha curcas* by expressed sequence tag (EST) sequencing of embryo cDNA libraries and rapid amplification of cDNA ends (RACE). *JcHSP-1* and *JcHSP-2* contained open-reading frames encoding sHSPs of 219 and 157 amino acids, with predicted molecular weights of 24.42 kDa and 18.02 kDa, respectively. Sequence alignment indicated that both JcHSP-1 and JcHSP-2 shared high similarity with other plant sHSPs. Real-time quantitative RT-PCR analysis showed that the transcriptional level of both *JcHSP-1* and *JcHSP-2* increased along with natural dehydration process during seed development. A sharp increase of *JcHSP-2* transcripts occurred in response to water content dropping from 42% in mature seeds to 12% in dry seeds. Western blot analysis revealed that the accumulation profile of two cross-reacting proteins, whose molecular weight corresponding to the calculated size of JcHSP-1 and JcHSP-2, respectively, was well consistent with the mRNA expression pattern of *JcHSP-1* and *JcHSP-2* in jatropha seeds during maturation and natural dehydration. These results indicated that both JcHSPs might play an important role in cell protection and seed development during maturation of *J. curcas* seeds.

1. Introduction

Small heat shock proteins (sHSPs) are a family of low molecular mass proteins (15–42 kDa) [1], which play an important role in the defense of organisms against a variety of environmental stresses and in various developmental processes [2]. sHSPs are the most abundant heat stress-induced proteins in higher plants where they were induced as molecular chaperones that play a protective role against various stresses such as cold, salt, drought and oxidants besides high temperature [3–7]. Moreover, many sHSPs appear to be also involved in various plant developmental processes, such as pollen development [8], embryo development [9,10], seed maturation [11,12], as well as cell proliferation and differentiation [13]. Three *Arabidopsis* sHSPs (AtHsp17.4, AtHsp17.6 and AtHsp17.7) accumulated during the middle stage of seed maturation, and their concentration remained high during the late stage and in mature dry seeds [12]. Similarly, rice Oshsp16.9A abundantly accumulated in mature dry seeds [14]. *Arabidopsis* mutant plants sensitive to desiccation contained lower amounts of sHSPs during seed maturation [15]. The synthesis of sHSPs during seed maturation suggests their probable role involved in the desiccation tolerance. As well as late embryogenesis abundant proteins (LEA) accumulated during seed maturation and acquisition of desiccation tolerance [16].

Plant sHSPs are all encoded by nuclear multigene families and classified into six subfamilies. Three subfamilies of sHSPs are localized to cytosol, and the other three organelle sHSP subfamilies localized to the chloroplast (CP), the endoplasmic reticulum (ER) and the mitochondria (MT), respectively [17,18]. The common feature of most sHSPs contains a conserved C-terminal alpha-crystallin domain (ACD) of approximately 90 amino acid residues, which is homologous to alpha-crystallin proteins of the vertebrate eye lens [19]. The structural organization of sHSPs is evolutionarily con-

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served, and the proteins of the same class from different plant species show a high sequence similarity [20].

Jatropha curcas L. (hereafter refer as jatropha) belongs to the genus *Jatropha* of the family *Euphorbiaceae* and mainly distributes in tropical and subtropical regions of South America and South Asia. It can be used as a biodiesel feedstock plant, a source of medicine products and biological pesticides, and for soil and water conservation and land reclamation [21]. For its high content of seed oil (up to 40%) [22] that has the similar characteristics to those of fossil diesel [23] and its strong adaptability to the drought and impoverished soil, jatropha is considered a promising biodiesel feedstock plant [24,25]. Some genes encoding fatty acid biosynthesis enzymes and affecting seed oil contents in jatropha have been cloned [26,27]. However, the cloning and characterization of sHSPs during jatropha seed maturation has not yet been reported.

In this study, we cloned two full-length cDNAs encoding sHSPs from developing jatropha seeds, designated *JcHSP-1* and *JcHSP-2*, by the approaches of expressed sequence tag (EST) sequencing of embryo cDNA libraries and rapid amplification of cDNA ends (RACE). Sequence analysis revealed that *JcHSP-1* and *JcHSP-2* shared high similarity with sHSPs from other plants. Their expression patterns during maturation and natural desiccation processes of jatropha seeds were investigated by real-time quantitative RT-PCR (qRT-PCR) analysis and western blot analysis.

2. Materials and methods

2.1. Plant materials

J. curcas L. seeds were collected from a local population in the Xishuangbanna Tropical Botanical Garden (XTBG), Chinese Academy of Sciences, Yunnan Province, PR China. When the plants came into blossom, the fruits development was recorded throughout the fruiting period. The whole seeds at different developmental stages (50, 55, 60, 65, 70, 90 and 100 days after flowering (DAF)) were harvested, flash frozen in liquid nitrogen and stored at −80 °C for molecular analysis.

2.2. Fresh and dry weight and water content analysis

A random sampling of fifteen seeds at different developmental stages was weighed to determine the fresh weight (FW). The dry weight (DW) of seeds was determined by reweighing after oven drying for 17 h at 103 °C. Water content (WC, %) was calculated based on FW and DW. Three independent replicates of fifteen seeds were used for individual stages.

2.3. Heat shock protein preparation and western blot analysis

Seeds at different developmental stages were ground to fine powder in liquid nitrogen. The powder was transferred immediately to a 10-ml tube containing extraction buffer [50 mM phosphate, pH 7.0, 0.2% (v/v) Triton-X-100, 7 mM β-mercaptoethanol and 5 mM ascorbic acid]. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was transferred to fresh tube and incubated at 100 °C for 10 min, then the tube was transferred to ice for 5 min. The heat-treated supernatant was centrifuged at 12,000 rpm for 15 min, and 50–100 μl of supernatant was transferred to new tubes for direct use or storage at −80 °C. Concentrations of heat-stable proteins were determined according to the Bradford protocol [28]. SDS–PAGE was performed with a discontinuous buffer system, as described by Laemmli [29]. Protein samples in 1× SDS gel-loading buffer were denatured by heated at 95 °C for 5 min before loaded into the gel. Fifteen micrograms of protein samples were separated electrophoretically on low range SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) microporous membranes (Millipore, Cat. No. IPVH00010) using the Trans-Blot®cell (Bio-Rad). The western blotting procedures were carried out according to Mazhar and Basha [30] using anti-sHSPs antibody (anti-AtHsp17.6 class I, kindly supplied by Dr. E. Vierling, University of Tucson, Arizona, USA) [12] and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Vector Laboratories, CA 94010, USA). Fresh-developing buffer (100 μl of NBT solution and 100 μl of BCIP solution in 10 ml of alkaline phosphatase buffer) [31] was used for membrane staining, and the reaction was stopped by washing the membrane in ddH_2O.

2.4. Cloning of jatropha sHSP cDNAs

Two sHSP unigene sequences [GenBank FM894116 for *JcHSP-1* and GenBank FM891678 for *JcHSP-2*] were obtained from the EST database of jatropha embryo cDNA libraries (GenBank FM887038-FM896881). Total RNA was extracted from jatropha seeds at different developmental stages using silica particle protocol [32]. Contaminated DNA was removed by incubating at 37 °C for 30 min with DNase I. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm. Reverse transcription-polymerase chain reaction (RT-PCR) was used to clone sHSP cDNAs with primers derived from sHSP EST sequences. The 3′ ends of sHSP cDNA were obtained by using 3′ Rapid Amplification of cDNA Ends (3′-RACE) according to the protocol of Rapid Amplification of cDNA Ends (Clontech). The 3′-RACE products were purified and sequenced.

2.5. qRT-PCR analysis

First-strand cDNA was obtained from 2 μg total RNA, M-MLV reverse transcriptase (Promega), dNTP minxture, and Oligo-d(T)18 primer (TaKaRa) incubating at 42 °C for 60 min in a 10 μl reaction volume. Then 2 μl cDNA sample diluted at 1:5 (v/v) was used as template for quantitative RT-PCR of 20 μl reaction volume. Real time quantitative RT-PCR (qRT-PCR) were performed with LightCycler® 480 Instrument (Roche) and LightCycler® 480 Relative Quantification Software. *JcHSP-1* specific primers (XT68, 5′-ATGGCGTACTCGGTTGCTCTTAA-3′; XT69, 5′-GT-GTTCTGTTCCA-CGGAGACCTT-3′) and *JcHSP-2* specific primers (XT5, 5′-TTCC-CTTCATCTTCTTCCCTCGTC-3′; XT6, 5′-TGAACTTGCCACTGCTACG-CTCC-3′) were designed based on our jatropha EST sequences of *JcHSP-1* (GenBank FM894116) and *JcHSP-2* (GenBank FM891678) using Primer Premier v5.0 (Premier Biosoft International). Jatropha actin gene (GenBank FM894455) was used as an internal control for the relative amount of RNA, which was amplified with the gene specific primers XT24 (5′-TGCAGACCGTATGAGCAAGGAGATC-3′) and XT25 (5′-CCA-GAGGGACCATTACAGTTGAGCC-3′). Only a single peak presented in each of the melting curves for amplification of *JcHSP-1*, *JcHSP-2* and *actin*, respectively, demonstrating that all the primer pairs used were specific.

Total 20 μl of reaction mixture containing the following components: 1× TransStart Green qPCR SuperMix, primers (0.25 mM each of them), 1× Passive Reference Dye/PCR Enhancer (50×) and 2 μl of the first-strand cDNAs diluted at 1:5 (v/v). Amplifications were performed under the following thermal cycling parameters: pre-denaturation at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 54–59 °C (a specific annealing temperature for each gene) for 50 s and 72 °C for 50 s, and followed by 1 cycle of final elongation at 72 °C for 7 min. Amplification-product specificity was checked with a heat-dissociated protocol (melting curves in 56–90 °C) following the final step of PCR. The efficiency-corrected quantification was performed automatically by the LightCycler® 480 Relative Quantification Software based on relative standard curves, which described